High Pressure Liquid Chromatography of Benzodiazepines: Analysis of Ketazolam

D. J. WEBER

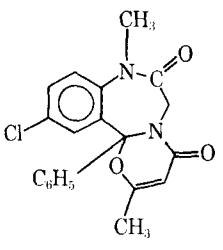
Abstract □ The analysis of ketazolam is complicated by the fact that there is a very slow release of diazepam in a nonaqueous system. High pressure liquid chromatography, a rapid room temperature separation process, appears uniquely useful for this analysis since TLC is difficult and GC procedures are not quantitative or are unable to separate ketazolam from the breakdown products. Results of the analysis indicate that the solid-state stability of ketazolam is good. The rate of ketazolam degradation in a nonaqueous, aprotic solvent was measured and shown to be a first-order process with a half-life of 59 hr. at 26°. Evidence is given showing that diazepam is probably the sole degradation product in the nonaqueous solvent. Data showing the detection sensitivity of diazepam and ketazolam are given, and a comparison with some other benzodiazepines is made.

Keyphrases □ Ketazolam—half-life, separation from diazepam, analysis, high pressure liquid chromatography □ Benzodiazepines—separation, analysis, high pressure liquid chromatography □ High pressure liquid chromatography—analysis, ketazolam—, diazepam, nitrazepam, oxazepam

A wide variety of psychotherapeutic drugs based on the benzodiazepine structure have been tested and a number of them have been marketed. Some of the marketed drugs are diazepam, oxazepam, and nitrazepam. Another highly active benzodiazepine of therapeutic interest is ketazolam.

The GC of benzodiazepines was previously reported (1, 2); it required no derivative formation and used simple, rapid, extraction procedures to isolate the drug from serum or urine. The amount injected onto the column ranged from a few nanograms (1) to several hundred nanograms (2). The high pressure liquid chromatography of some benzodiazepines was also reported (3). However, the true potential of the technique was not demonstrated since the amounts injected were in the microgram range and the mobile phase used caused some deterioration of the stationary phase. Other analytical techniques for benzodiazepines suffer from a lack of specificity (4–6) or are too slow (7).

An attempt at GC analysis of ketazolam showed[1] that the drug immediately pyrolyzed to produce diazepam. Thus, a mixture of ketazolam and diazepam gave a single diazepam peak and no analysis was possible. Since diazepam is used as the starting material in the synthesis of ketazolam (8), it is important to have available an analytical procedure that can rapidly differentiate ketazolam from diazepam. This paper describes a liquid chromatographic analytical procedure for separating ketazolam from diazepam and also gives a better estimate of the ultimate sensitivity of high pressure, high performance liquid chromatographic analysis of some benzodiazepines.



ketazolam

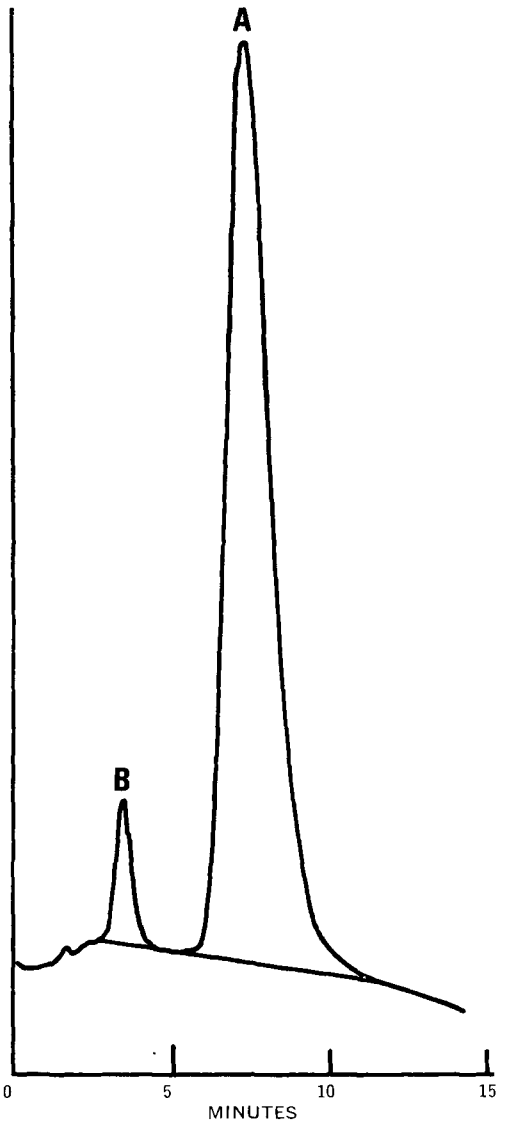


Figure 1—*Typical liquid chromatogram of (A) 590 ng. of ketazolam, and (B) 24 ng. of diazepam on a Corasil II column with a 15% tetrahydrofuran–85% isopropyl ether mobile phase. Sensitivity is 0.02 absorbance unit full-scale.*

[1] F. S. Eberts, Jr., private communication, The Upjohn Co., Kalamazoo, MI 49001

Table I—Peak Height of Diazepam as a Function of Nanograms Injected

Nanograms Injected	Microliters Injected	Peak[a] Height, mm.
8.65	1	34.0
		33.5
		34.5
17.30	2	69.0
		68.0
25.95	3	105.0
		106.5
43.26	1	160.0
		162.0
86.53	2	350.0
		340.0
129.79	3	528.0
		530.0
216.32	1	817.6
		739.2
432.64	2	1688.0
		1680.0
648.96	3	2568.0
		2568.0

[a] Calculated at a sensitivity of 0.005 absorbance unit full-scale deflection.

EXPERIMENTAL

Materials—Samples of diazepam[2], oxazepam[3], and nitrazepam[2] were obtained commercially and used as received. Two samples of micronized ketazolam, prepared by the literature method (8), had the following analysis.

Anal.—Calc. for $C_{20}H_{17}ClN_2O_3$: C, 65.13; H, 4.65; Cl, 9.61; N, 7.60. Found: C, 65.17, 65.19; H, 4.64, 4.69; Cl, 9.78, 9.65; N, 7.70, 7.59.

Liquid Chromatography—Approximately 5.0-mg. samples of each benzodiazepine were accurately weighed into 25-ml. volumetric flasks, dissolved in sufficient mobile phase, and diluted to the mark. The time for 50% dissolution of ketazolam was estimated to be about 1.0 min. and was recorded as zero time in the rate studies. The tetrahydrofuran[4] (I) was a special grade packed under nitrogen and it contained no antioxidant. The isopropyl ether[5] (II) contained a small amount of UV absorbing antioxidant.

The liquid chromatograph[6] was equipped with 1-m. columns. A Corasil II[7] column was used for the separation of ketazolam and diazepam, and a Durapak-OPN/Poracil C[7] column was used for the chromatography of diazepam, oxazepam, and nitrazepam alone. The mobile phases were 10% I plus 90% II for the OPN/Poracil C column and 15% I plus 85% II for the Corasil II column work. The flow rates were 1.0 (Corasil II) and 1.25 (OPN/Poracil C) ml./min. under a pressure of 200 p.s.i. Samples of 1–4 μl. were injected with a 10-μl. syringe[8] using the stop-flow technique. All measurements were made at room temperature (26°). Detection was by UV absorbance at 254 nm.

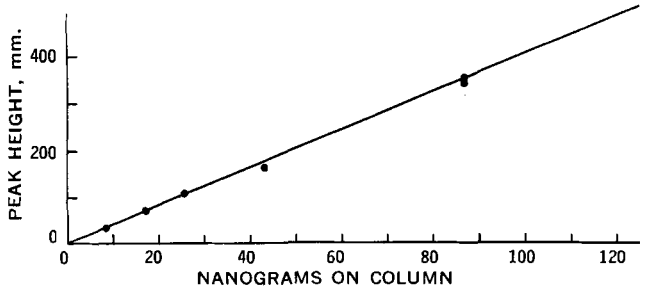


Figure 2—*High pressure liquid chromatography of diazepam. Column = Corasil II; mobile phase = 15% tetrahydrofuran–85% isopropyl ether, pressure = 200 p.s.i., and sensitivity = 0.005 absorbance unit full-scale.*

[2] Hoffmann–La Roche.
[3] Wyeth.
[4] Burdick and Jackson Labs.
[5] Union Carbide.
[6] Model 820, DuPont Instrument Products.
[7] Waters Associates.

Table II—Liquid Chromatographic Analysis of Diazepam Content of Ketazolam Clinical Batches[a] as a Function of Time

Run	Nanograms Injected	Microliters Injected	Diazepam Peak Height, mm.	Nanograms of Diazepam	Minutes
		Lot B			
1	654.12	3	37.0	8.3	4.0
2	654.12	3	46.7	10.6	18.0
3	654.12	3	51.0	11.6	33.0
4	654.12	3	66.0	15.2	56.0
		Lot A			
1	635.76	3	72.7	16.7	7.0
2	635.76	3	78.5	18.1	20.0
3	635.76	3	86.2	19.8	33.0
4	635.76	3	93.0	21.6	47.0

[a] The loss of ketazolam during the time of analysis was: B, 1.17% (0.0209%/min.); and A, 0.94% (0.0199%/min.).

Rate Studies—Since ketazolam slowly breaks down to diazepam in the tetrahydrofuran–isopropyl ether solvent system it was necessary to assay repeatedly the test solutions as a function of time and to extrapolate the results to zero time. Solutions were prepared as previously described and analyzed using the Corasil II column. Injections of 1-, 2-, or 3-μl. samples of the degrading solution were made and the results were analyzed kinetically.

RESULTS AND DISCUSSION

Chromatography of Ketazolam and Diazepam—An example of a typical separation of ketazolam from diazepam is shown in Fig. 1. The retention times of diazepam and ketazolam are 3.5 and 7.5 min., respectively. The linearity of a plot of diazepam peak height *versus* nanograms on column is shown in Fig. 2. Additional data in Table I indicate that the calibration curve is linear to at least 650 ng. The data in Fig. 2 indicate that about 5 ng. of diazepam on column is the lower limit of measurement. A plot of nanograms of ketazolam *versus* peak height is shown in Fig. 3. The response is linear but is less sensitive than for diazepam. The lower limit of measurement is about 30 ng. The scatter of the points in Fig. 3 is due to the fact that the stock standard solution of ketazolam in the mobile phase is not stable and must be continually corrected for the increasing diazepam content.

Analysis of Diazepam Content of Ketazolam—The data given in Table II and the example plotted in Fig. 4 indicate that the initial rate of conversion of ketazolam to diazepam is linear and approximately equal for both samples, as would be expected for first-order

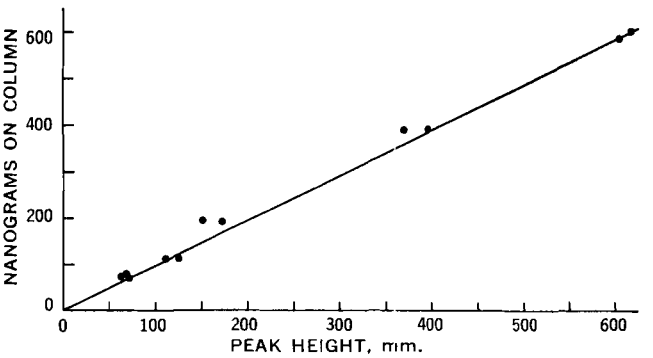


Figure 3—*Plot of nanograms of ketazolam on column* versus *peak height. The ordinate was corrected for diazepam content.*

[8] Precision Sampling Corp.

Table III—Diazepam Content of Ketazolam as a Function of Time

Minutes	Peak Height of Diazepam, mm.
0	26.0[a]
40	43.0
55	54.0
115	85.0
130	96.0
145	97.2
159	102.0
177	103.0
193	108.0
241	136.5
261	144.5
275	160.0
288	187.5
301	172.5
315	176.8
1767	616.8
1783	660.0
3215	937.6
∞	2023.0[b]

[a] The initial content of diazepam represents 1.06% of the sample. [b] This is a calculated value assuming complete conversion to diazepam.

degrading solutions of nearly equal concentrations. The extrapolated zero-time diazepam contents of ketazolam Lots A and B are 2.48 and 1.18%, respectively. The A and B samples were 1 and 2 years old, respectively, and had been stored in closed bottles at room temperature. Despite the instability of ketazolam in the tetrahydrofuran–isopropyl ether mobile phase, it appears that the drug is quite stable in the solid state.

Products and Rate of Conversion of Ketazolam—The data in Table II, plotted as in Fig. 4, have essentially the same slopes which are equal to 0.13 and 0.12 ng./min. These slopes represent the initial rates of conversion of ketazolam to diazepam and are equal to 0.0210 and 0.0199%/min., respectively. Therefore, at these concentrations and in this particular solvent system, less than a 0.5% change in ketazolam content would be expected to occur during the 20 min. needed for the injection and elution of a single sample. This change is acceptable for most analyses of ketazolam content of a sample, but accurate analysis of a minor component such as diazepam requires multiple assays as a function of time and extrapolation to zero time.

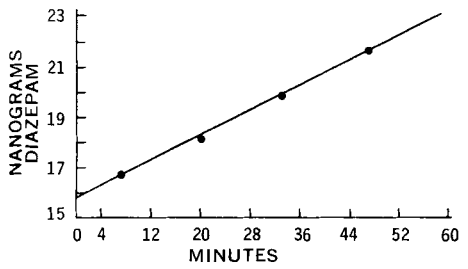


Figure 4—*Plot of diazepam content* versus *time for ketazolam, Lot A. Slope is 0.12 ng./min. Diazepam content at* t_0 *is 2.48%.*

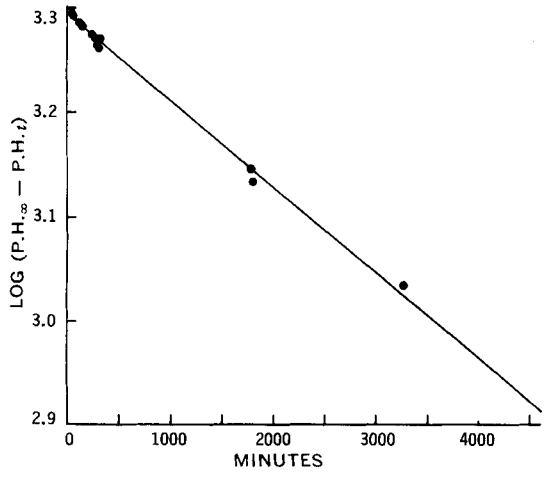


Figure 5—*First-order plot of formation of diazepam from ketazolam in 15% tetrahydrofuran–85% isopropyl ether at 26°. The rate constant is 1.94 × 10⁻⁴ min.⁻¹ and the half-life is 59.6 hr.*

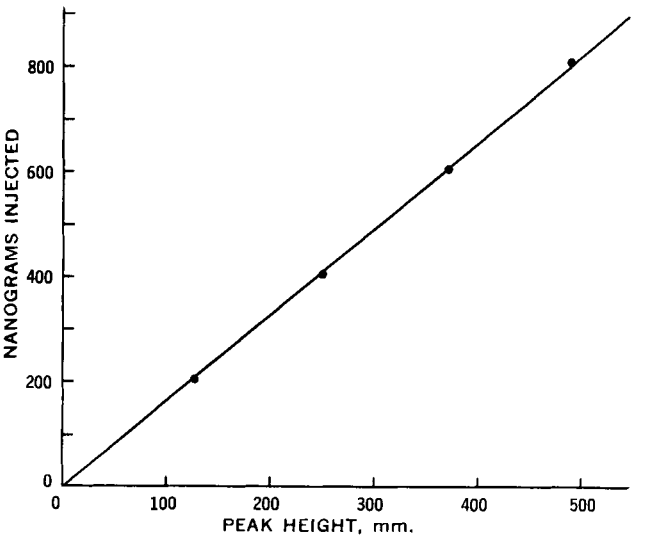


Figure 6—*High pressure liquid chromatography of nitrazepam. Column = OPN-Poracil C, mobile phase = 10% tetrahydrofuran–90% isopropyl ether, pressure = 200 p.s.i., and sensitivity = 0.005 absorbance unit full-scale.*

Two aged solutions of ketazolam in 15% I–85% II were analyzed for residual ketazolam content. A 46-day-old sample (55.0 mcg./ml.) showed no residual ketazolam, and the peak of the formed diazepam was 97% of the height expected assuming a 1:1 conversion of ketazolam to diazepam. A 3-day-old sample (214.7 mcg./ml.) in the same solvent showed 43.6% of the ketazolam still remaining. The height of the formed diazepam peak was 94% of the height expected for 1:1 conversion of ketazolam to diazepam. These data indicate that, under the experimental conditions, the breakdown of ketazolam results in the formation of diazepam and that further degradation of diazepam probably does not occur.

A sample of ketazolam different from Lots A and B was dissolved in 15% I–85% II and analyzed for the initial diazepam con-

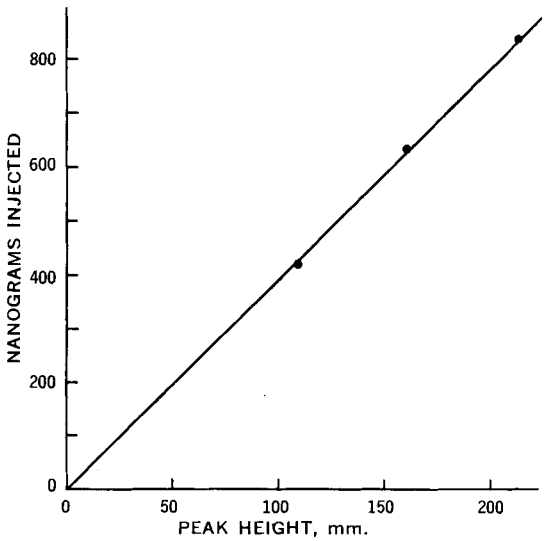


Figure 7—*High pressure liquid chromatography of oxazepam. Column = OPN-Poracil C, mobile phase = 10% tetrahydrofuran–90% isopropyl ether, pressure = 200 p.s.i., and sensitivity = 0.005 absorbance unit full-scale.*

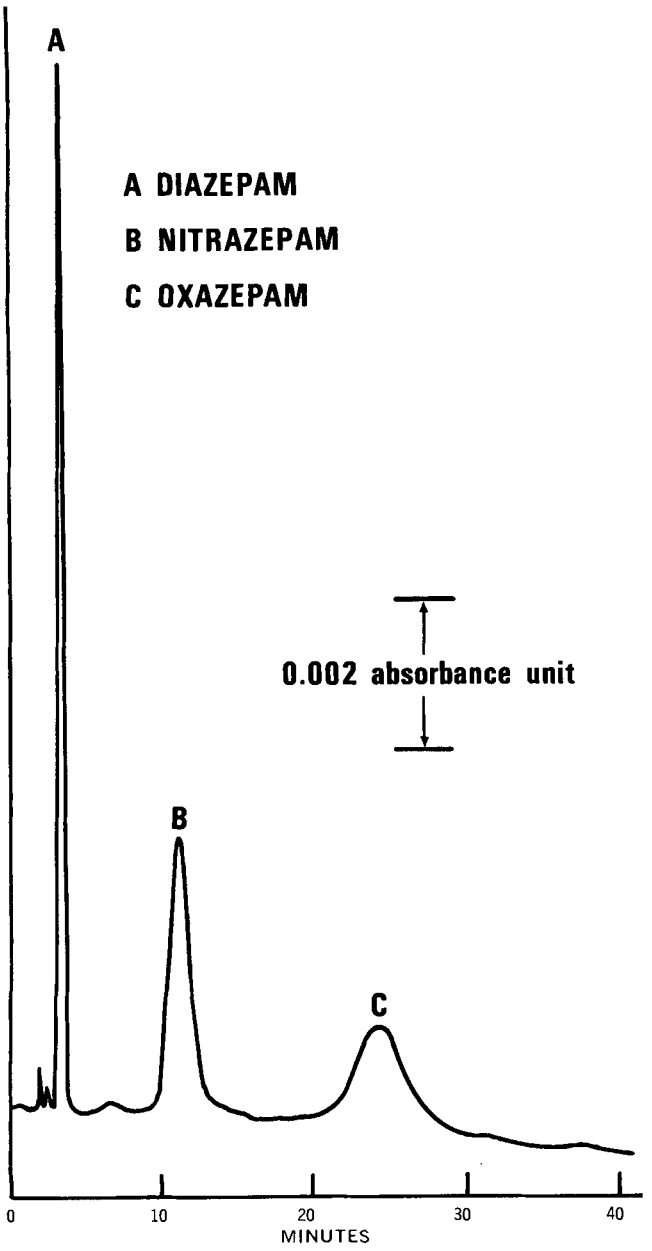


Figure 8—*Separation of diazepam, nitrazepam, and oxazepam using high pressure liquid chromatography.*

tent and the rate of diazepam increase. The results are given in Table III. A plot of the initial data of Table III gave a slope of 0.0191%/min., which is in excellent agreement with the values found for Lots A and B. The intercept of the initial data was 26 mm. and represents 1.06% diazepam in the original sample. A semilog plot of the data in Table III is shown in Fig. 5. The slope of the line corresponds to a first-order rate constant of 1.94×10^{-4} min.$^{-1}$ and a half-life of 59.6 hr. at 26°.

Since the 15% I–85% II solvent system is a dry, nonacidic, neutral solvent, acid or base catalysis is not occurring and the observed decomposition is probably due to spontaneous elimination of the elements of diketene resulting in the formation of diazepam.

Chromatography of Nitrazepam and Oxazepam—The data in Figs. 2 and 3 indicate that about 5 ng. of diazepam and 30 ng. of ketazolam on column are the lower limits of measurement (10-mm. peak) for the two drugs. This represents nearly a 1000-fold increase in sensitivity when compared with the results previously reported (3). Extension of these results to nitrazepam and oxazepam gave the results shown in Figs. 6 and 7. The plots are linear over a wide range and the limits of measurement are 15 and 40 ng. for nitrazepam and oxazepam, respectively. The sensitivity of measurement of both nitrazepam and oxazepam can be increased by raising the concentration of I in the mobile phase. This results in a smaller retention time and a higher peak. An example of the separation of diazepam, nitrazepam, and oxazepam is shown in Fig. 8.

SUMMARY

The separation and analysis of samples containing ketazolam and diazepam were described, demonstrating the properties of speed, sensitivity, and gentle treatment of samples by high pressure liquid chromatography. Data were given showing that ketazolam breaks down to diazepam, with a half-life of 59 hr., in a nonaqueous, dry, aprotic solvent at 26°. The lower limit of on-column measurement of benzodiazepines was shown to be in the very low nanogram range, in contrast to previous literature data which seemed to indicate that microgram quantities were needed for injection. The separation of diazepam, nitrazepam, and oxazepam using a stable column–mobile phase combination was described.

REFERENCES

(1) F. Marcucci, R. Fanelli, and E. Mussini, *J. Chromatogr.*, **37**, 318(1968).

(2) L. B. Foster and C. S. Frings, *Clin. Chem.*, **16**, 177(1970).

(3) C. G. Scott and P. Bommer, *J. Chromatogr. Sci.*, **8**, 446(1970).

(4) J. A. F. DeSilva, M. A. Schwartz, V. Stefanovic, J. Kaplan, and L. D'Arconte, *Anal. Chem.*, **36**, 2099(1969).

(5) G. Caille, J. Braun, and J. A. Mockle, *Can. J. Pharm. Sci.*, **5**, 78(1970).

(6) J. Braun, G. Caille, and E. A. Martin, *ibid.*, **3**, 65(1968).

(7) S. Lauffer and E. Schmid, *Arzneim.-Forsch.*, **19**, 740(1969).

(8) J. Szmuskovicz, C. G. Chidester, D. J. Duchamp, F. A. Mak-Kellar, and G. Slomp, *Tetrahedron Lett.*, **1971**, 3665.

ACKNOWLEDGMENTS AND ADDRESSES

Received May 12, 1972, from the *Research Laboratories, The Upjohn Company, Kalamazoo, MI 49001*

Accepted for publication July 5, 1972.